Brush-Border-Enzyme-Mediated Intestine-Specific Drug Delivery. Amino Acid Prodrugs of 5-Aminosalicylic Acid

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5-Aminosalicylic acid (5-ASA) is the active principle of a number of preparations aimed at the treatment of inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, but its efficacy is limited by early absorption and metabolism. The possibility to exploit the selective hydrolytic activity of brush border enzymes such as aminopeptidase A and carboxypeptidases was studied by preparing the following four amino acid prodrugs of 5-ASA: 5-(*N*-L-aspartylamino)-2-salicylic acid, disodium salt (**18**), 5-(*N*-L-glutamylamino)-2-salicylic acid, disodium salt (**19**), [(5-aminosalicyl)-L-prolyl]-L-leucine, sodium salt (**25**), and [[5-(*N*-L-glutamylamino)salicyl]-L-prolyl]-L-leucine, disodium salt (**28**). In these compounds, the peptide bond is selectively split by the intestinal brush border aminopeptidase A (compounds **18**, **19**, and **28**) and carboxypeptidases (compounds **25** and **28**).

Introduction

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), remain a problem to be solved by the biomedical community. Centered on the treatment with corticosteroids and salicylates, the medical therapy of IBD is still empirical due to an inadequate knowledge of the pathogenesis and a lack of understanding about the etiology of the disease.[1,2] Corticosteroids such as the newer fluticasone,[3] beclometasone,[4] and budenoside[5] are believed to exert their effects by stimulating the endogenous phospholipase A_2 inhibitor lipocortin, thus impairing the release of arachidonic acid from membrane phospholipids.[6] Although effective for an attack therapy of ulcerative colitis, corticosteroids are disappointing when used in moderate doses as maintenance therapy to prevent relapses. On the other hand, larger doses cannot be justified for long-term treatment because of the risk of serious side effects such as osteoporosis, hypertension, fluid retention, increased susceptibility to infection, and glycosuria.[7]

Since its initial use by the Swedish physician Nana Svartz in the early 1940s,[8,9] sulfasalazine (SASP, **1**), an azo-bond conjugate of sulfapyridine (SP, **2**) and 5-aminosalicylic acid (5-ASA, **3**), has become the most widely prescribed agent for IBD. Clinical studies have shown that SASP is beneficial in mild and moderately active IBD and is the drug of choice for the maintenance therapy enforced to prevent relapses. When orally ingested, about 70% of SASP reaches the distal small intestine and colon[10] where it is split by azo reductases of the colonic microflora to release 5-ASA, the active principle, and SP (Figure 1).

5-ASA remains largely in the colon, and about 70% of it is excreted unchanged in the feces.[11] The rest is partly absorbed and partly excreted in the urine as an *N*-acetyl derivative. Most SP is absorbed from the colon and excreted in the urine in the free form or as *N*-acetyl and glucuronide derivatives. Although generally safe, SASP cannot be given to more than 80% of patients because of hypersensitivity or less specific forms of intolerance.[11-13] Hypersensitivity is characterized by skin rash, arthritis, pericarditis, and pancreatitis, among other reactions. As nearly all the side effects of SASP are induced by SP, the development of sulfapyridine-free 5-ASA derivatives has largely been explored.[14,15] Since 5-ASA is rapidly absorbed in the proximal small intestine and either excreted in the urine or returned to the bile in acetylated form before

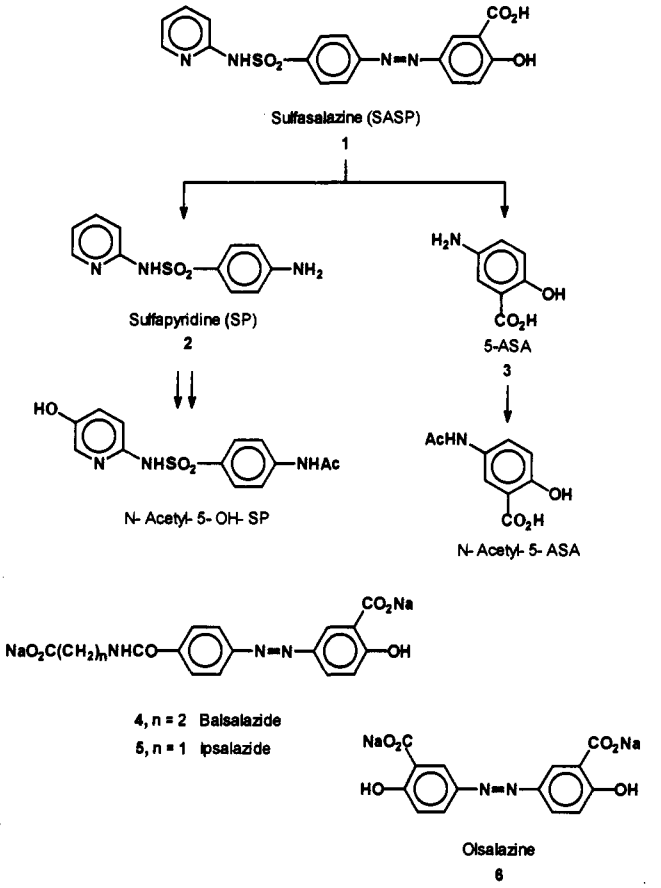

Figure 1. Sulfasalazine (SASP) metabolic pathway.

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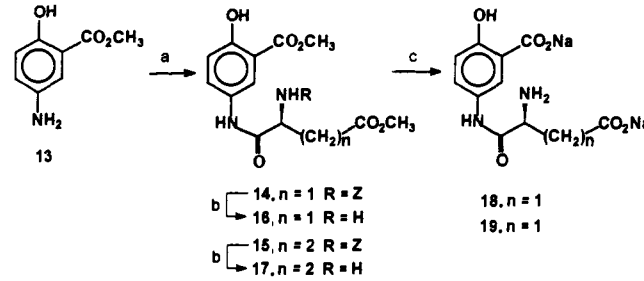
reaching the ileum and colon,[16] two main approaches aimed at delivering intact 5-ASA to the lower intestine, as SASP does, have been explored. Firstly, several slow-release or sustained-release formulations have been developed, and all coat and buffer 5-ASA in a different way: Salofalk (5-ASA buffered with sodium carbonate and glycine in an enteric soluble film), Asacol, Claversal (5-ASA coated with Eudragit, an acrylic-based resin), and Pentasa (5-ASA in microcapsules with an ethyl cellulose membrane).[17] Secondly, 5-ASA has been coupled via an azo link to a variety of carriers such as an inert polymeric backbone,[18] (4-aminobenzoyl)-β-alanine[19] (Balsalazide, 4), (4-aminobenzoyl)glycine[19] (Ipsalazide, 5), or 5-ASA itself (Olsalazine, 6).[20] Recently, the synthesis of *N*- and *O*-glycopyranosyl derivatives of 5-ASA has been reported.[21] The colon-specific delivery of 5-ASA from these prodrugs relies on the glycosidase activity of gut microflora, according to a strategy already applied to corticosteroids.[22]

We describe now a new approach for the intestinal-specific delivery of 5-ASA, based on the selective cleavage operated on amino acid prodrugs by enzymes localized in the intestinal brush border,[23] a functional organelle situated at the luminal pole of the enterocyte, mainly destined to the terminal digestion and absorption of the end products of ingested food. Different classes of brush border enzymes have been identified, among them phosphatases involved in the release of inorganic phosphates of the diet,[24] disaccharidases involved in carbohydrate digestion,[25] and peptidases having an analogous role in protein digestion and characterized by functional selectivity and by a distinct distribution along the gastrointestinal tract.[26,27] The possibility to exploit the specificities of these enzymes for an amino-acid-based site-specific drug delivery system has been first proposed by Amidon.[28-30] Among the host of brush border peptidases, in particular, aminopeptidase A is able to hydrolyze peptides having an acidic N-terminal amino acid such as aspartic or glutamic acid, while carboxypeptidase is able to cleave C-terminal residues from peptides having proline in the penultimate position. We have examined the possibility to utilize this selectivity of peptide-bond cleavage exhibited by brush border enzymes for the intestinal-specific delivery of 5-ASA. On the basis of the above rationale, we have synthesized four novel prodrugs of 5-ASA (3): 5-(*N*-L-aspartylamino)-2-salicylic acid, disodium salt (18), 5-(*N*-L-glutamylamino)-2-salicylic acid, disodium salt (19), [(5-aminosalicyl)-L-prolyl]-L-leucine, sodium salt (25), and [[5-(*N*-L-glutamylamino)salicyl]-L-prolyl]-L-leucine, disodium salt (28). The L-glutamate and L-aspartate derivatives 18 and 19, respectively, were designed to release 5-ASA following cleavage of the peptide bond by aminopeptidase A. The prolyl–leucyl dipeptide 25 was designed to release 5-ASA following cleavage by carboxypeptidase. With the aim to prolong even further the release of 5-ASA in the distal part of the intestine, finally, the two approaches where combined in compound 28.

The aim of our study was to determine (a) the selectivity of cleavage of these compounds by intestinal enzymes and, consequently, the extent of release of 5-ASA at the level of the small intestine; (b) the availability of 5-ASA released from these new prodrugs compared with that of SASP and free 5-ASA; and (c) whether colostomy influences the extent of metabolism of the prodrugs and the absorption of the metabolites.

Scheme I[a]



[a] Conditions: (a) 7 or 8, DCC, imidazole, AcOEt; (b) H_2, 5% Pd/C, MeOH, 50 psi; (c) i. 1 N NaOH, ii. reversed-phase medium-pressure chromatography.

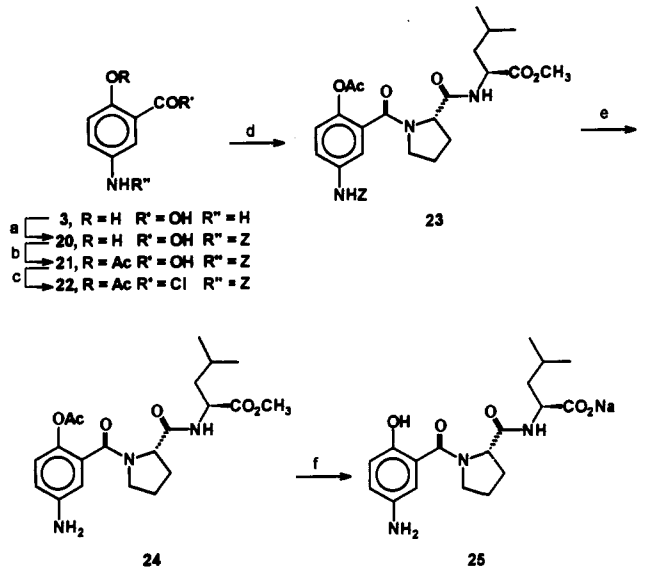
Chemistry

5-(*N*-L-Aspartylamino)salicylic acid, disodium salt (18) and 5-(*N*-L-glutamylamino)salicylic acid, disodium salt (19) were synthesized as described in Scheme I. Condensation of 5-ASA methyl ester (13)[31] with β-methyl *N*-(benzyloxycarbonyl)-L-aspartate (7)[32,33] using DCC and imidazole afforded methyl 5-[*N*-[methyl *N*-(benzyloxycarbonyl)-L-aspartyl ester]amino]salicylate (14) in 75% yield. Removal of the *N*-benzyloxycarbonyl group from 14 was achieved by catalytic hydrogenolysis (5% Pd/C in MeOH, 50 psi), thus obtaining methyl 5-[*N*-(methyl L-aspartyl ester)amino]salicylate (16) in 91% yield. Hydrolysis with 1 N NaOH and purification by reversed-phase chromatography afforded 5-(*N*-L-aspartylamino)-salicylic acid, disodium salt (18) in 94% yield. Analogously, condensation of 13 with γ-methyl *N*-(benzyloxycarbonyl)-L-glutamate (8)[32,33] in the same conditions as above gave methyl 5-[*N*-[methyl *N*-(benzyloxycarbonyl)-L-glutamyl ester]amino]salicylate (15) in 75% yield. Removal of the *N*-benzyloxycarbonyl group from 15 (H_2, 5% Pd/C in MeOH, 50 psi) afforded methyl 5-[*N*-(methyl L-glutamyl ester)amino]salicylate (17) in 95% yield, which was then hydrolyzed with 1 N NaOH and purified by reversed-phase chromatography to give 5-(*N*-L-glutamylamino)-salicylic acid, disodium salt (19) in 63% yield.

Synthesis of [(5-aminosalicyl)-L-prolyl]-L-leucine, sodium salt (25; Scheme II) involved the initial preparation of 5-[*N*-(benzyloxycarbonyl)amino]-2-acetoxysalicyl chloride (22) and L-prolyl-L-leucine methyl ester (12). 5-ASA (3) was reacted with benzyl chloroformate in a saturated $NaHCO_3$ solution to give the corresponding Z derivative 20 in 92% yield. Treatment of 20 with pyridine and acetic anhydride gave the acetyl derivative 21 in 70% yield which was then reacted with thionyl chloride in pyridine, thus affording the corresponding acyl chloride 22 in 81% yield.

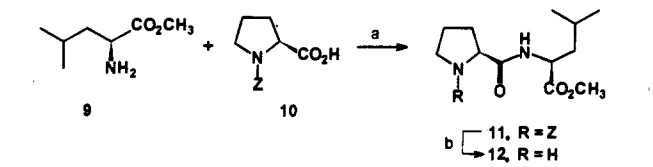
L-Prolyl-L-leucine 12 was prepared as depicted in Scheme III. Thus, condensation of L-leucine methyl ester (9)[34] and *N*-(benzyloxycarbonyl)-L-proline (10)[35] in the presence of DCC and imidazole afforded [*N*-(benzyloxycarbonyl)-L-prolyl]-L-leucine methyl ester (11) in 96% yield, which was then submitted to catalytic hydrogenolysis (10% Pd/C in MeOH, 50 psi) to give the corresponding dipeptide 12 in 99% yield. When the acyl chloride 22 and the dipeptide 12 were condensed in refluxing CCl_4, [[5-[*N*-(benzyloxycarbonyl)amino]-2-acetoxysalicyl]-L-prolyl]-L-leucine methyl ester (23) was obtained in 57% yield. Catalytic hydrogenolysis (5% Pd/C in MeOH, 50 psi) of 23 afforded [(5-amino-2-acetoxysalicyl)-L-prolyl]-L-leucine methyl ester (24) in 80% yield, which was hydrolyzed in 1 N NaOH to give the corresponding [(5-aminosalicyl)-L-prolyl]-L-leucine, sodium salt (25) in 76% yield.

Scheme II[a]



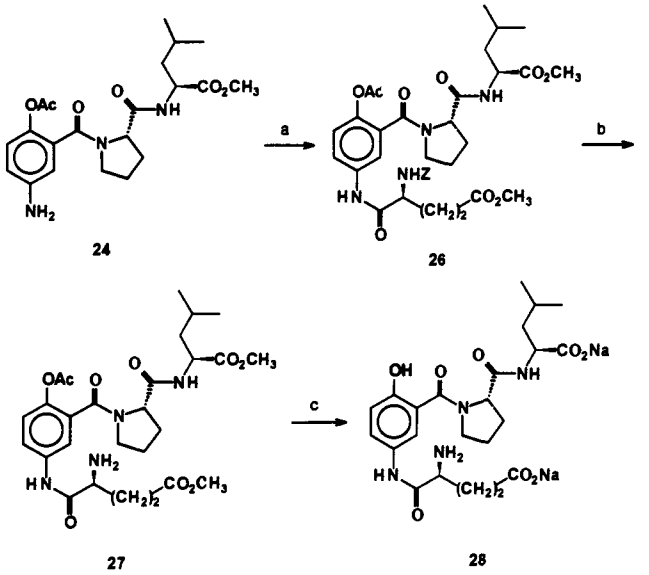
[a] Conditions: (a) ZCl, $NaHCO_3$; (b) Py, Ac_2O, AcOH; (c) $SOCl_2$, Py, C_6H_6; (d) **12**, CCl_4, reflux; (e) H_2, 5% Pd/C, MeOH, 50 psi; (f) i. 2 NaOH, ii. reversed-phase medium-pressure chromatography.

Scheme III[a]



[a] Conditions: (a) DCC, imidazole, AcOEt; (b) H_2, 10% Pd/C, MeOH, 50 psi.

Scheme IV[a]



[a] Conditions: (a) **8**, DCC, imidazole, CH_2Cl_2; (b) H_2, 10% Pd/C, MeOH, 50 psi; (c) i. 2 N NaOH; ii. reversed-phase medium-pressure chromatography.

The diester **24** was also used as the starting product for the preparation of [[5-(*N*-L-glutamylamino)salicyl]-L-prolyl]-L-leucine, disodium salt (**28**; Scheme IV). Condensation of **24** with γ-methyl *N*-(benzyloxycarbonyl)-L-glutamate (**8**)[32,33] in the presence of DCC and imidazole gave the protected tripeptide **26** in 60% yield. Catalytic hydrogenolysis (10% Pd/C in MeOH, 50 psi) of **26** afforded **27** in 99% yield, which was then hydrolyzed with 2 N NaOH to give the corresponding disodium salt **28** in 52% yield.

Results and Discussion

In Vivo Experiments. In order to test the release of 5-ASA in the terminal part of the small intestine, we have performed a series of experiments in which compounds **18**, **19**, **25**, and **28** were administered orally to male Fisher colostomized and noncolostomized rats and compared to 5-ASA and SASP. Urines and feces of the animals were collected at 2-h intervals for 48 h and examined for their content in 5-ASA and its metabolite, *N*-acetyl-5-ASA. The colostomy was performed to assess the concentrations of 5-ASA and *N*-acetyl-5-ASA at the level of the proximal part of the ascending colon, the intestinal tract, that together with the terminal ileum is most often involved in Crohn's disease[36] as well as in severe forms of ulcerative colitis.[37] The administration of the compounds was done in noncolostomized animals to verify if colostomy influences the extent of the metabolism of the prodrugs and the absorption of the metabolites. Results of these experiments are shown in Table I and Figure 2 for the cumulative amounts (5-ASA + *N*-acetyl-5-ASA) detected in both urines and feces and in Figure 3 which represents the time-dependent curves observed in urines and feces, respectively, after oral administration of compounds **18**, **19**, **25**, and **28** as compared to that of 5-ASA (**3**) and SASP (**1**). No significant differences were found between colostomized and noncolostomized animals for 48-h cumulative fecal and urinary excretion of the compounds 5-ASA and *N*-acetyl-5-ASA after administration of all the compounds studied (Figure 2). After the administration of 5-ASA, there was a high urinary excretion ((78.8 ± 6.9)%) of 5-ASA and *N*-acetyl-5-ASA, showing a high absorption of 5-ASA in the first tract of the small intestine as previously described,[38] and this is confirmed by its low fecal excretion ((4.3 ± 2.1)%). After the administration of SASP, the fecal recovery of 5-ASA and *N*-acetyl-5-ASA was higher with respect to free administered 5-ASA, showing a better release of the drug in the large intestine. All the new prodrugs of 5-ASA gave a high fecal recovery of metabolites with respect to 5-ASA administered in free form, although there was a different behavior among these new prodrugs. Compound **28** showed the highest fecal recovery ((48.9 ± 4.1)%), even compared to SASP, while compound **25** produced a low fecal recovery ((22.9 ± 4.1)%). It is important to note that in all cases the total amount recovered from urines and feces is ca. 75%, never reaching 100%. This confirms earlier observations[39] and may account for the presence of other metabolites such as glucuronides[40] not taken into consideration in this work. Moreover, 5-ASA and its *N*-acetyl derivative might be attached to the ileocolonic mucosa[41] and not measured in the feces. Particular attention should be focused on the unexpected low value of recovery from the feces observed for compound **25**. We hypothesize two different conditions: (a) an early release of 5-ASA due to the intervention of gastric or pancreatic peptidases or (b) a different distribution of carboxypeptidases and aminopeptidase along the gastrointestinal tract. In fact, human carboxypeptidases[27] are homogeneously distributed from the ligament of Treitz to the distal ileum. Therefore, they may release 5-ASA from **25** at a proximal part with following absorption, thus explaining its relatively low concentration in the colon. On the contrary, the concen-

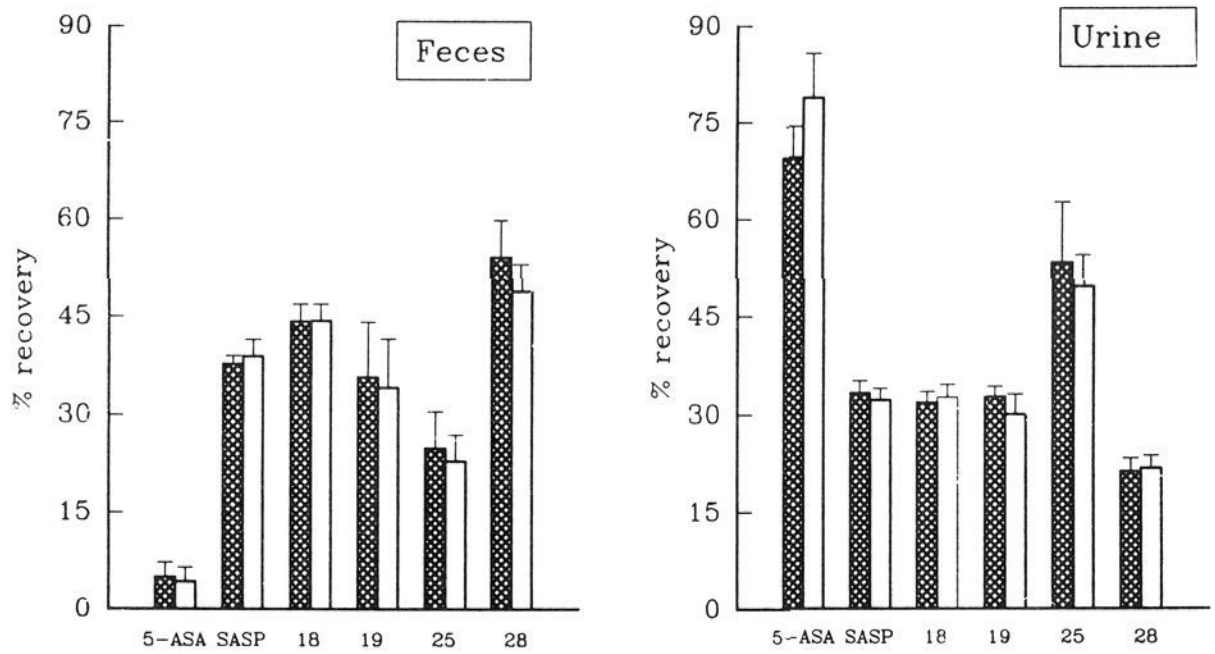


Figure 2. Percent recovery of 5-ASA and *N*-acetyl-5-ASA in urines or feces 48 h after administration of 5-ASA (**3**), SASP (**1**), (*N*-L-aspartylamino)-2-salicylic acid **18**, (*N*-L-glutamylamino)-2-salicylic acid **19**, [(5-aminosalicyl)-L-prolyl]-L-leucine **25**, and [[5-(*N*-L-glutamylamino)salicyl]-L-prolyl]-L-leucine **28** in colostomized rats (shaded columns) and in normal animals (unshaded columns).

Table I. Cumulative Fecal and Urinary Recovery of 5-ASA and *N*-Acetyl-5-ASA Expressed as Mean ± SD in Colostomized and Noncolostomized Rats

	fecal recovery				urinary recovery			
	n	colostd (%)	noncolostd (%)	$P < 0.05$	*n*	colostd (%)	noncolostd (%)	$P < 0.05$
5-ASA (**3**)	5	5.08 ± 2.24	4.32 ± 2.14	NS	5	69.61 ± 4.87	78.84 ± 6.90	NS
SASP (**1**)	5	37.77 ± 1.29	38.90 ± 2.51	NS	5	33.34 ± 1.90	32.21 ± 1.85	NS
18	5	35.73 ± 8.35	34.20 ± 7.45	NS	5	32.66 ± 1.63	30.09 ± 3.18	NS
19	5	44.20 ± 2.70	44.29 ± 2.58	NS	5	31.83 ± 1.77	32.42 ± 2.08	NS
25	5	24.98 ± 5.61	22.94 ± 4.10	NS	5	53.22 ± 9.44	49.60 ± 4.87	NS
28	5	54.10 ± 5.79	48.90 ± 4.15	NS	5	21.22 ± 2.13	21.70 ± 2.25	NS

tration of aminopeptidases in the brush border increases along the gastrointestinal tract reaching its highest value in the ileum,[27] allowing a more distal release of 5-ASA from compounds **18**, **19**, and **28**.

One major concern about the present strategy was that patients suffering from IBD might have a dysfunctioning brush border,[42] the system on which our approach is based. Although this point needs to be confirmed by administration to patients with IBD, it can be anticipated that aminopeptidases from these patients do not present any changes in their cytoplasmic activity.[43]

In Vitro Experiment. The hydrolysis of compound **19** occurred only after incubation in a solution of peptidases; the reaction appeared to follow first-order kinetics with a $T_{1/2}$ of 81 min (Figure 4). The incubation of compound **19** with gastric juice, pancreatic juice, bovine proteases, and fresh human fecal suspension did not result in the release of 5-ASA and *N*-acetyl-5-ASA.

This experiment indicates that only peptidases are able to hydrolyze **19** in vitro.

Conclusions

The results so far obtained indicate that amino acid derivatives of 5-ASA such as **18**, **19**, **25**, and **28** can find useful application for the controlled release of 5-ASA in the intestinal lumen. The four amino acid derivatives of 5-ASA exhibit a different metabolic profile. Compounds **18** and **19** are cleaved by aminopeptidase A and show a metabolic profile similar to that of SASP. Compounds **18**, **19**, and **28** can be particularly useful when Crohn's disease or ulcerative rectocolitis is localized in the terminal ileum or in the right portion of the large intestine. Finally, the early release of 5-ASA, characterizing compound **25**, can be advantageously exploited for Crohn's disease or ulcerative rectocolitis forms affecting the proximal and distal portions of the small intestine. Further studies aimed at characterizing the therapeutic potential of compounds **18**, **19**, **25**, and **28** are in progress.

Experimental Section

General Methods. Melting points were determined on a Kofler micro-hot-stage apparatus and are uncorrected. IR spectra were recorded with a Perkin-Elmer 1320 spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were taken on a Bruker AC 200 spectrometer, and the chemical shifts are in ppm downfield from tetramethylsilane, except with D_2O which was used without an internal standard. Flash chromatography was performed on Merck silica gel (0.040–0.063 mm). Medium-pressure chromatography was performed on Merck LiChroprep Si 60 and LiChroprep RP-8 (reversed-phase) lobar columns.

[*N*-(Benzyloxycarbonyl)-L-prolyl]-L-leucine Methyl Ester (11). A solution of DCC (7.8 g, 37.8 mmol) in anhydrous AcOEt (40 mL) was added dropwise in 15 min to a stirred solution of **9** (5.0 g, 34.4 mmol) and **10** (8.5 g, 34.4 mmol) in anhydrous AcOEt (110 mL) containing imidazole (2.3 g, 34.4 mmol). Stirring was continued overnight, after which the reaction mixture was filtered and the filtrate concentrated in vacuo. The residue (13 g) was submitted to flash chromatography; elution with $CHCl_3$ gave **11** (12.3 g, 96%): ^{1}H NMR ($CDCl_3$) δ 0.85–1.00 (d, 6H, Me_2CH), 1.45–1.75 (m, 3H, Me_2CHCH_2), 1.80–2.40 (m, 4H, C-3 and C-4 CH_2), 3.40–3.60 (m, 2H, C-5 CH_2), 3.70 (s, 3H, CO_2Me), 4.30–4.60 (2 × m, 2H, 2 × CHCO), 5.20 (s, 2H, $PhCH_2$), 7.25–7.40 (m, 5H, ar).

L-Prolyl-L-leucine Methyl Ester (12). A solution of **11** (6.0 g, 15.93 mmol) in MeOH (75 mL) was reacted with 10% Pd/C and H_2 in a Parr apparatus at a pressure of 50 psi for 45 min. The catalyst was then filtered over Celite and the filtrate evaporated under reduced pressure to give **12** as a yellowish oil (3.85 g, 99.5%): ^{1}H NMR ($CDCl_3$) δ 0.90–1.00 (2 × d, 6H, Me_2CH), 1.45–2.45 (br m, 7H, Me_2CHCH_2 and C-3 and C-4 CH_2), 3.45 (s, 3H, CO_2Me), 3.50–3.65 (m, 2H, C-5 CH_2), 3.95–4.20 (m, 2H, 2 × CHCO), 7.10 (br s, 1H, CH_2–*NH*).

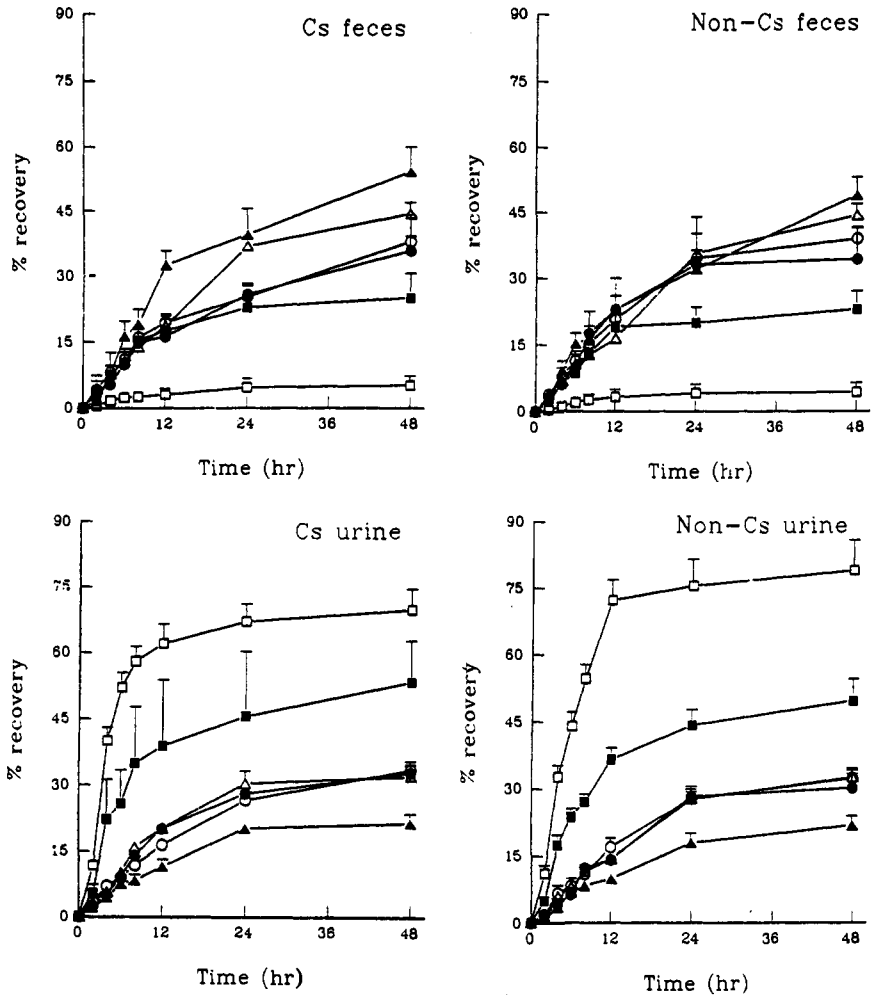


Figure 3. Analysis of the time charts of fecal and urinary recovery of 5-ASA and *N*-acetyl-5-ASA after administration of 5-ASA (**3**) (□), SASP (1) (○), (*N*-L-aspartylamino)-2-salicylic acid **18** (●), (*N*-L-glutamylamino)-2-salicylic acid **19** (△), [(5-aminosalicyl)-L-prolyl]-L-leucine **25** (■), and [[5-(*N*-L-glutamylamino)salicyl]-L-prolyl]-L-leucine **28** (▲) in colostomized (Cs) and in noncolostomized (Non-Cs) rats.

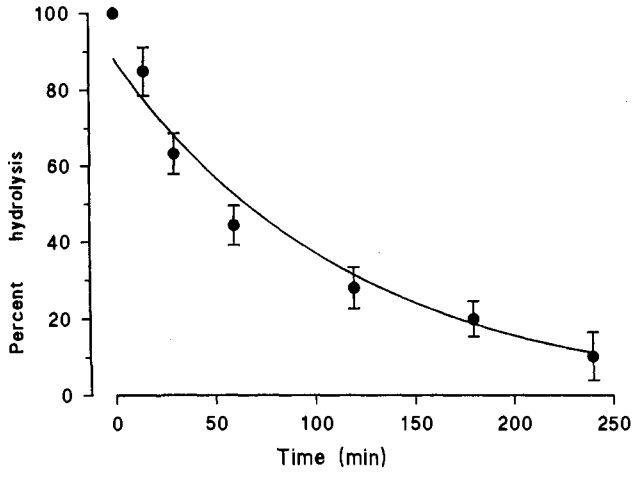


Figure 4. Hydrolysis of (*N*-L-glutamylamino)-2-salicylic acid **19** by peptidase.

Methyl 5-[*N*-[Methyl *N*-(benzyloxycarbonyl)-L-aspartyl ester]amino]salicylate (14) and Methyl 5-[*N*-[Methyl *N*-(benzyloxycarbonyl)-L-glutamyl ester]amino]salicylate (15). A solution of DCC (2.04 g, 9.0 mmol) in EtOAc (50 mL) was added dropwise in 4 h to a magnetically stirred solution of **7** (2.53 g, 8.9 mmol) and **13** (1.5 g, 8.9 mmol) in EtOAc (50 mL) containing imidazole (0.61 g, 8.9 mmol), and the resulting mixture was allowed to react overnight. The white precipitate thus obtained was then filtered and the filtrate evaporated in vacuo. The residue (3.5 g) was purified by flash chromatography using $CHCl_3$–MeOH 99:1 as the eluent to yield **14** as a white solid (2.9 g, 75%), mp 104–106 °C: ^1H NMR ($CDCl_3$) δ 2.75–3.05 (m, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 4.65–4.80 (m, 1H, CH), 5.15 (s, 2H, OCH_2), 6.20 (d, 1H, NH), 6.90 (d, 1H, ar), 7.30–7.50 (m, 6H, ar), 8.00 (d, 1H, ar), 8.55 (s, 1H, NH).

An analogous procedure was employed to prepare **15** from **8**. In this case, final purification by flash chromatography using $CHCl_3$ as the eluent yielded **15** (75%) as a white solid, mp 108–110 °C: ^1H NMR ($CDCl_3$) δ 1.90–2.70 (m, 4H, CH_2–CH_2), 3.65 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 4.30–4.50 (m, 1H, CH), 5.10 (s, 2H, OCH_2), 5.95 (d, 1H, NH), 6.85 (d, 1H, ar), 7.20–7.50 (m, 6H, ar), 8.05 (d, 1H, ar), 8.70 (s, 1H, NH).

Methyl 5-[*N*-(Methyl L-aspartyl ester)amino]salicylate (16) and Methyl 5-[*N*-(Methyl L-glutamyl ester)amino]-salicylate (17). Pd/C (5%, 250 mg) was added to a suspension of **14** (2.4 g, 8.1 mmol) in MeOH (40 mL), and the resulting mixture was submitted to H_2 in a Parr apparatus at a pressure of 50 psi for 6 h. The reaction mixture was then filtered under Celite to remove the catalyst and the filtrate evaporated in vacuo to give **16** (1.5 g, 91%), mp 83–84 °C: ^1H NMR ($CDCl_3$) δ 2.85 (s, 2H, NH_2), 2.80–3.05 (m, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.80 (dd, 1H, CH), 3.95 (s, 3H, OCH_3), 6.95 (d, 1H, ar), 7.60 (dd, 1H, ar), 8.15 (d, 1H, ar), 9.45 (s, 1H, NH).

Analogously was prepared **17** from **15**. The final product was recrystallized from ethyl acetate–hexane to give **17** (95%), mp 175–177 °C: ^1H NMR (D_2O) δ 2.20 (m, 2H, CH_2), 2.55 (t, 2H, CH_2), 3.55 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 4.10 (t, 1H, CH), 6.75 (d, 1H, ar), 7.35 (dd, 1H, ar), 7.70 (d, 1H, ar).

5-(*N*-L-Aspartylamino)salicylic Acid, Disodium Salt (18) and 5-(*N*-L-Glutamylamino)salicylic Acid, Disodium Salt (19). A solution of **16** (2.0 g, 5.68 mmol) in 1 N NaOH (50 mL)

was kept under magnetic stirring and a nitrogen atmosphere for 6 h at room temperature. The reaction mixture was then neutralized and the solvent evaporated under reduced pressure. The residue (1.8 g) was submitted to medium-pressure chromatography on a reversed-phase column; elution with H_2O–MeOH 90:10 afforded **18** (1.5 g, 94%): ^{1}H NMR (D_2O) δ 2.70–2.95 (m, 2H, CH_2), 3.80–3.95 (m, 1H, CH), 6.70 (d, 1H, ar), 7.15 (dd, 1H, ar), 7.55 (d, 1H, ar). Anal. ($C_{11}H_{10}O_6N_2Na_2$) C, H, N.

Analogously, from **17** was prepared **19** (63%): ^{1}H NMR (D_2O) δ 1.90–2.55 (m, 4H, CH_2–CH_2), 4.20–4.35 (m, 1H, CH), 6.75 (d, 1H, ar), 7.25 (dd, 1H, ar), 7.60 (d, 1H, ar). Anal. ($C_{12}H_{12}O_6N_2Na_2$) C, H, N.

5-[*N*-(Benzyloxycarbonyl)amino]salicylic Acid (20). Benzyl chloroformate (36.7 g, 0.215 mol) was added dropwise in 40 min to a suspension of 5-aminosalicylic acid (**3**; 30 g, 0.20 mol) in a saturated solution of $NaHCO_3$ (500 mL) also containing solid $NaHCO_3$ (10 g), and the solution was magnetically stirred at 0 °C. Stirring was continued for 5 h, after which the precipitate thus formed was filtered and the filtrate was washed with Et_2O (3 × 50 mL), acidified with 3 N HCl, and extracted with AcOEt (3 × 100 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and, after evaporation of the solvent, afforded a solid which was added to the previously obtained precipitate to give **20** (53 g, 92%), mp 224–225 °C: ^{1}H NMR (DMSO-d_6) δ 5.17 (s, 2H, CH_2Ph), 7.00–8.05 (m, 8H, ar), 9.80 (s, 1H, CO_2H).

5-[*N*-(Benzyloxycarbonyl)amino]-2-acetoxysalicylic Acid (21). Pyridine (1.39 mL) and Ac_2O (35.5 g, 0.34 mol) were added to a stirred suspension of **20** (53 g, 0.184 mol) in AcOH (280 mL). Stirring was continued for 2 h, after which the resulting precipitate was filtered and dried under vacuum to yield **21** (40.1 g, 70%), mp 202–204 °C: ^{1}H NMR (MeOH-d_4) δ 2.15 (s, 3H, CO–CH_3), 5.10 (s, 2H, CH_2Ph), 6.90–8.00 (m, 8H, ar).

5-[*N*-(Benzyloxycarbonyl)amino]-2-acetoxysalicyl Chloride (22). A stirred suspension of **21** (35 g, 0.106 mol), $SOCl_2$ (2.52 g, 0.21 mol), and pyridine (1 mL) in anhydrous benzene (150 mL) was refluxed for 3 h in a nitrogen atmosphere. The reaction mixture was then cooled to obtain a white precipitate which was filtered and dried in vacuo to give **22** (29.89 g, 81%).

[[5-[*N*-(Benzyloxycarbonyl)amino]-2-acetoxysalicyl]-L-prolyl]-L-leucine Methyl Ester (23). A stirred solution of **22** (28 g, 0.08 mol) and **12** (19.4 g, 0.08 mol) in anhydrous CCl_4 (300 mL) was refluxed for 12 h in a nitrogen atmosphere. The reaction mixture was then filtered and the filtrate evaporated in vacuo to give a residue which was submitted to flash chromatography; elution with $CHCl_3$ afforded **23** (25 g, 57%): ^{1}H NMR ($CDCl_3$) δ 0.95 (2d, 6H, CH(CH_3)$_2$), 1.50–2.15 (m, 10H, CO–CH_3, NCH_2–CH_2CH_2, $CHMe_2$), 3.45 (s, 3H, CO_2CH_3), 3.70 (m, 2H, 2 × NH), 4.20–4.70 (m, 2H, 2 × CH), 5.10 (d, 2H, CH_2Ph), 6.90–8.00 (m, 8H, ar).

[(5-Amino-2-acetoxysalicyl)-L-prolyl]-L-leucine Methyl Ester (24). A solution of **23** (1.7 g, 3.0 mmol) in MeOH (11 mL) was reacted with 5% Pd/C and H_2 in a Parr apparatus at a pressure of 50 psi overnight. The catalyst was then filtered over Celite and the filtrate evaporated under reduced pressure to give **24** (1.0 g, 80%), mp 83–84 °C; ^{1}H NMR ($CDCl_3$) δ 0.95 (2d, 6H, CH(CH_3)$_2$), 1.60–2.35 (m, 10H, CO–CH_3, $NCH_2CH_2CH_2$, $CHMe_2$), 3.60–4.80 (m, 6H, CO_2CH_3, 2 × CH, NH), 6.90–8.00 (m, 3H, ar).

[(5-Aminosalicyl)-L-prolyl]-L-leucine, Sodium Salt (25). A solution of **24** (5.0 g, 12 mmol) in 1 N NaOH (15 mL) was kept under magnetic stirring and a nitrogen atmosphere for 6 h at room temperature. The reaction mixture was then acidified to pH 5.0, and, after concentration under reduced pressure, the residue was submitted to medium-pressure chromatography on a reversed-phase column; elution with H_2O–MeOH 90:10 afforded **25** (3.2 g, 76%): ^{1}H NMR (D_2O) δ 0.95 (2d, 6H, CH(CH_3)$_2$), 1.60–2.30 (m, 7H, $NCH_2CH_2CH_2$, $CHMe_2$), 4.20 (m, 2H, 2 × CH), 6.90–7.20 (m, 3H, ar). Anal. ($C_{17}H_{22}O_5N_3Na$) C, H, N.

[[5-[*N*-[Methyl *N*-(benzyloxycarbonyl)-L-glutamyl ester]-amino]-2-acetoxysalicyl]-L-prolyl]-L-leucine Methyl Ester (26). DCC (1.4 g, 7.8 mmol) was added portionwise to a magnetically stirred solution of γ-methyl *N*-(benzyloxycarbonyl)-L-glutamate (**8**; 2.12 g, 7.18 mmol) and **24** (3 g, 7.15 mmol) in anhydrous CH_2Cl_2 (10 mL) containing imidazole (0.49 g, 7.2 mmol), and the resulting mixture was allowed to react at room temperature overnight. The precipitate thus formed was filtered and the filtrate evaporated under reduced pressure to give a residue which was submitted to flash chromatography; elution with $CHCl_3$–MeOH 90:10 yielded **26** (3.1 g, 60%): ^{1}H NMR ($CDCl_3$) δ 0.85 (2d, 6H, CH(CH_3)$_2$), 1.35–2.50 (m, 14H, $CHMe_2$, $COCH_3$, CO(CH_2)$_2$–CH, $NCH_2CH_2CH_2$), 3.50–4.10 (m, 6H, 2 × CO_2CH_3), 4.15–4.75 (m, 3H, 3 × CH), 5.10 (d, 2H, CH_2Ph), 6.20–7.80 (m, 8H, ar).

[[5-[*N*-(Methyl L-glutamyl ester)amino]-2-acetoxysalicyl]-L-prolyl]-L-leucine Methyl Ester (27). A solution of **26** (0.30 g, 0.431 mmol) in MeOH (15 mL) was reacted with 5% Pd/C and H_2 in a Parr apparatus at a pressure of 50 psi for 4 h. The catalyst was then filtered over Celite and the filtrate evaporated under reduced pressure to give **27** (0.24 g, 99%), mp 159–161 °C: ^{1}H NMR ($CDCl_3$) δ 0.85 (2d, 6H, CH(CH_3)$_2$), 1.35–2.50 (m, 14H, $CHMe_2$, $NCH_2CH_2CH_2$, CO(CH_2)$_2$–CH, $COCH_3$), 3.50–4.10 (m, 6H, 2 × CO_2CH_3), 4.15–4.75 (m, 3H, 3 × CH), 6.90–7.60 (m, 3H, ar).

[[5-(*N*-L-Glutamylamino)salicyl]-L-prolyl]-L-leucine, Disodium Salt (28). A stirred solution of **27** (0.2 g, 0.29 mmol) in 2 N NaOH (10 mL) was kept under argon for 6 h at room temperature. The reaction mixture was then neutralized with 3 N HCl, and, after concentration under reduced pressure, the residue was submitted to medium-pressure chromatography on a reversed-phase column; elution with water gave **28** (0.10 g, 52%): ^{1}H NMR (D_2O) δ 0.85 (2d, 6H, CH(CH_3)$_2$), 1.37–2.30 (m, 11H, $CHMe_2$, CO(CH_2)$_2$–CH, $NCH_2CH_2CH_2$), 4.15–4.40 (m, 3H, 3 × CH), 6.90–7.60 (m, 3H, ar). Anal. ($C_{22}H_{28}O_8N_4Na_2$) C, H, N.

Statistical Analysis. Data are presented as combined cumulative recovery of 5-ASA and *N*-acetyl-5-ASA in feces and urine, expressed as the percent of the amount of 5-ASA administered in free form or in each of the compounds. The results are given as mean ± SD. Data were analyzed with a professional statistics software program[44] as a multivariate four-factor factorial experiment. The crossed factors were defined as colon (colostomized–noncolostomized), excretion (urine and feces), and drugs (six drugs). Time was the repeated measure factor (each subject was analyzed at seven different times). The main effects and interactions were assessed at the 0.05 level of significance.

Biological Tests. Materials. 5-ASA (99% purity) and SASP (95% purity) were purchased from Sigma-Chemical (Milan, Italy). Human gastric juice (pH = 2) was obtained endoscopically from healthy volunteers. The peptidases from porcine intestinal mucosa (activity 115 units/g of solid) were purchased from Sigma-Chemical (Milan, Italy). Proteases derived from bovine pancreas were obtained also from Sigma-Chemical (activity (8–10) units/mg of solid). Human pancreatic juice was obtained by duodenal drainage from healthy volunteers, and the chymotryptic activity (90 IU/mL) was measured by the method of Figarella.[45] Human feces were collected from healthy volunteers.

In Vivo Experiment. Male Fisher rats (200–250 g, n. 60) were supplied by Charles River, Como, Italy, and fed with Intralipid and a 5% glucose solution for 2 days to prepare the animal for surgery. The animals were anesthetized with ip injections of Nembutal (7.5 mg/kg of body weight). In 30 animals, a median laparotomy was then performed and colostomy was made 2 days after maintaining the animals exclusively on a liquid diet of 5% glucosate solution and 10% Intralipid (100 g of soy lipids, 12 g of egg yolk phospholipids; Pierrel, Naples, Italy). After the operation, the animals were fed a strictly liquid diet for 2 days and then a normal commercial diet was introduced (type 4 RF 21; Mucedola, Settimo Milanese, Italy). After a week from surgery, the animals were ready for the experiment. After the animals had fasted for 12 h, the compounds were administered by an intragastric probe at a concentration of 60 mg/kg equiv of 5-ASA suspended in 2.5% arabic gum. The animals were then placed in metabolic cages and their urines and feces collected separately at 2-h intervals for 48 h. The same protocol for drug administration and sample collection was carried out in normal intact animals (n. 30). The samples collected were immediately frozen at −20 °C before being analyzed by HPLC using the following procedure: 0.1 mL of urine or 0.1 g of feces was diluted with 0.9 mL of methanol. A 25-μL portion of the methanolic extract was injected onto a 25- × 0.46-cm ultrasphere O.D.S. column (particle size 0.5 μm). Liquid chromatography was carried out using a mobile phase consisting of 20% methanol and 80%

phosphate buffer (pH = 6.0) with 0.5 mM tetrabutylammonium chloride added and with a flow rate of 1.7 mL/min. Detection of 5-ASA and *N*-acetyl-5-ASA was assessed by UV absorption at 254 nm.[46]

In Vitro Experiment. In order to be successful, amino acidic prodrugs such as **18**, **19**, **25**, and **28** must have adequate stability under the variety of conditions characterizing the GI tract in order to avoid reconversion. With the aim to evaluate these parameters, we chose compound **19** as a model and submitted it to a variety of "in vitro" tests. Compound **19** (50 mg) was then dissolved and incubated (for 72 h at 37 °C) in (a) human gastric juice (15 mL) at pH = 2, (b) a suspension of fresh human feces (1 g) dissolved in distilled water (15 mL), (c) human pancreatic juice (15 mL) at pH = 7.8 containing 90 IU/mL of chymotryptic activity, (d) bovine protease (10 units) dissolved in distilled water (15 mL), and (e) porcine peptidases (1 and 10 units) dissolved in distilled water (15 mL). Portions of the samples were taken to determine the amounts of 5-ASA and *N*-acetyl-5-ASA that were released from the parent drug.

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